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Novel Attenuated Chikungunya Vaccine Candidates Elicit Protective Immunity in C57BL/6 mice

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ABSTRACT

Chikungunya virus (CHIKV) is a reemerging mosquito-borne alphavirus that has caused severe epidemics in Africa and Asia and occasionally in Europe. As of today, there is no licensed vaccine available to prevent CHIKV infection. Here we describe the development and evaluation of novel CHIKV vaccine candidates that were attenuated by deleting a large part of the gene encoding nsP3 or the entire gene encoding 6K and were administered as viral particles or infectious genomes launched by DNA. The resulting attenuated mutants were genetically stable and elicited high magnitudes of binding and neutralizing antibodies as well as strong T cell responses after a single immunization in C57BL/6 mice. Subsequent challenge with a high dose of CHIKV demonstrated that the induced antibody responses protected the animals from viremia and joint swelling. The protective antibody response was long-lived, and a second homologous immunization further enhanced immune responses. In summary, this report demonstrates a straightforward means of constructing stable and efficient attenuated CHIKV vaccine candidates that can be administered either as viral particles or as infectious genomes launched by DNA.

IMPORTANCE

Similar to other infectious diseases, the best means of preventing CHIKV infection would be by vaccination using an attenuated vaccine platform which preferably raises protective immunity after a single immunization. However, the attenuated CHIKV vaccine candidates developed to date rely on a small number of attenuating point mutations and are at risk of being unstable or even sensitive to reversion. We report here the construction and preclinical evaluation of novel CHIKV vaccine candidates that have been attenuated by introducing large deletions. The resulting mutants proved to be genetically stable, attenuated, highly immunogenic, and able to confer durable immunity after a single immunization. Moreover, these mutants can be administered either as viral particles or as DNA-launched infectious genomes, enabling evaluation of the most feasible vaccine modality for a certain setting. These CHIKV mutants could represent stable and efficient vaccine candidates against CHIKV.

Chikungunya virus (CHIKV) is an arthropod-borne virus transmitted via *Aedes* mosquitoes and is known to cause severe arthralgic disease in humans. CHIKV belongs to the *Togaviridae* family, genus Alphavirus, and, in similarity to other alphaviruses, it carries a positive-sense single-stranded RNA genome of ~11 Kb containing two open reading frames encoding nonstructural proteins (nsP1 to nsP4) and structural proteins (C, E3, E2, 6K, and E1), respectively (1).

The first CHIKV infection was reported in Tanzania during the early 1950s (2) and was followed by sporadic outbreaks in tropical parts of Africa and Asia. CHIKV reemerged in 2005, causing severe epidemics on Indian Ocean Islands and later in both tropical and temperate countries of Africa and Asia and occasionally in Europe (3). For example, a massive one-third of the population on the French island La Reunion was infected during an outbreak in 2005 to 2006 (4), demonstrating the tremendous social and economic burden that can be caused by CHIKV epidemics. Consequently, CHIKV has been declared a Category C Priority Pathogen by the National Institute of Allergy and Infectious Diseases (NIAID) in the United States. The reemergence of CHIKV is thought to have been facilitated by a single amino acid shift in E1 (A226V), enabling replication and transmission of the virus by the aggressive and widespread *Aedes albopictus* mosquito (5, 6) in addition to the *Aedes aegypti* mosquito. Clinical manifestations of CHIKV infection include high fever, headache, rash, myalgia, and debilitating arthralgia (7). The infection typically resolves within weeks but can result in chronic joint problems (8, 9) or, rarely, mortality (10).

There is currently no CHIKV-specific treatment or approved vaccine to prevent CHIKV infection. A number of CHIKV vaccine candidates have been described, including attenuated (11–13) or inactivated (14, 15) CHIKV, alphavirus chimeras (16), and subunit (17–20) and genetic (21–24) vaccines. Since inactivated, subunit, and genetic vaccines typically require several immunizations to confer immunity and are expensive to produce, live-attenuated viruses are usually the most potent vaccines because of their ability to infect cells and stimulate both innate and adaptive immune responses, usually without the need for a booster immunization

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(25). One attenuated CHIKV vaccine candidate designated 181/clone25 developed by the U.S. Army has been evaluated clinically. Besides transient arthralgia detected in 8% of the vaccinees in a phase II clinical trial, the vaccine was well-tolerated and immunogenic (26). This vaccine has, however, not been further pursued due to virus passaging in uncertified cell cultures during development, and a more recent study indicated that 181/clone25 is attenuated by only two point mutations (27) and is at risk of reversion to pathogenic CHIKV. Similarly, other attenuated CHIKV vaccine candidates also rely on a small number of attenuating mutations (11–13).

To generate more-stable attenuated CHIKV vaccine candidates, we have attenuated the LR2006-OPY1 CHIKV infectious clone (28) either by deleting a large part of the gene encoding nsP3 or by deleting the entire gene encoding 6K. The nsP3 and 6K proteins are important for the function of the alphavirus replicase and for the formation and budding of new virions, respectively, and introducing the corresponding mutations into the closely related Semliki Forest virus (SFV) has been shown to reduce the replicative capacity of the resulting mutants (29, 30). Both vaccine candidates were produced and administered as viral particles or as DNA-launched infectious genomes. Initial studies *in vitro* revealed an excellent stability and attenuation profile of both vaccine candidates, and subsequent evaluation in a mouse infection model demonstrated that they were highly immunogenic after a single immunization and able to confer protection against challenge with a high dose of CHIKV. We therefore trust that the CHIKV mutants described here represent stable and efficient vaccine candidates against CHIKV.

MATERIALS AND METHODS

CHIKV and vaccine candidates. Construction of wild-type infectious clone CHIKV LR2006-OPY1, referred to here as CHIKV, has been previously described (28). Five viruses with deletions of different sizes in the hypervariable region of the nsP3 protein (mutants Δ1nsP3 to Δ5nsP3) were constructed, but only Δ5nsP3 viruses, having the largest deletion (amino acid [aa] residues 1656 to 1717 of the P1234 polyprotein were substituted with a linker [aa sequence AYRAAAG]), were used in this study. To obtain mutant virus lacking the 6K protein (referred to here as Δ6K), the sequence encoding aa residues 749 to 809 of the structural polyprotein was deleted. Plasmids for production of infectious virus stocks were generated by cloning cDNAs of CHIKV or mutant Δ5nsP3 or Δ6K under the control of an SP6 RNA polymerase promoter; and CHIKV and the mutants were produced as previously described (30, 31). Plasmid vectors capable of producing infectious viruses were constructed by cloning the cDNAs of the CHIKV, Δ5nsP3, or Δ6K strain under the control of the human cytomegalovirus immediate-early promoter in a previously used DNA-launched Semliki Forest virus replicon (DREP) plasmid (32). All resulting clones were verified by sequencing, and their sequences and cloning details are available from us upon request. UV-inactivated CHIKV was prepared by treating CHIKV in a UV cross-linker (600 mJ/cm^2) for 5 min. Inactivation was confirmed by examining cytopathic effects on baby hamster kidney (BHK-21) cells.

Stabilities of the Δ5nsP3 and Δ6K mutants were subsequently analyzed by serial passage of collected virus stocks five times in Vero cells at a multiplicity of infection (MOI) of 0.1 PFU/cell. For all passages, the stock titers were determined using plaque assay (see the "Viremia" section below) and the stability of introduced deletions was verified using reverse transcription-PCR (RT-PCR) and sequencing.

Setup of mouse challenge model. Female C57BL/6 mice (Charles River, Germany) were used for all experiments. To determine a suitable virus titer for challenge, 14-week-old mice were infected subcutaneously (s.c.) at the dorsal side of each hind foot with a total of 10^4, 10^5, or 10^6 PFU CHIKV diluted in 2× 20 μl phosphate-buffered saline (PBS). The same amounts of CHIKV diluted in 2× 50 μl PBS were injected s.c. in both flanks of 5- to 6-week-old mice, followed by challenge 7 weeks later with 10^6 PFU CHIKV inoculated into feet as described.

Immunizations. The vaccine candidates were tested in the challenge model using 5- to 6-week-old female C57BL/6 mice (5 mice/group). Mice were immunized with 10^4 or 10^5 PFU of either CHIKV or the Δ5nsP3 and Δ6K mutants or with the corresponding amount of UV-inactivated CHIKV diluted in 2× 50 μl PBS inoculated s.c. in both flanks or with 10 μg DNA encoding cDNA of CHIKV or the mutants diluted in 2× 20 μl PBS inoculated intradermally in both flanks. Uptake of injected DNA was augmented by *in vivo* electroporation using a DermaVax device as described previously (33). To study the pathogenicity of vaccine candidates as viral particles, 10^5 PFU was injected in the feet as described for the challenge. Serum samples were collected by tail bleedings and separation in Microtainer tubes (BD). All experiments were performed in at least two separate iterations.

Viremia. CHIKV titers of virus preparations and virus in serum collected at day 1 to day 3 postimmunization and at day 1 to day 3 postchallenge were determined by plaque assay. Serum was diluted in minimum essential medium (MEM) containing 0.2% bovine serum albumin (BSA) (Gibco/Life Technologies). Confluent BHK-21 cells in 6-well plates were washed with PBS twice, serum dilutions were added to the wells, and the wells were incubated at 37°C in 5% CO_2 for 1 h. The infection medium was removed, and 2 ml of overlay medium, consisting of 1 part MEM–1.8% agarose and 1 part complete BHK medium (BHK-21 Glasgow MEM supplemented with tryptose phosphate broth, 0.1 U/ml penicillin, 0.1 μg/ml streptomycin [strep], 2 mM L-glutamine, 10 mM HEPES, and 5% fetal bovine serum [Gibco/Life Technologies]), was added to each well. Plates were incubated at 37°C in 5% CO_2. After 48 h, 10% formalin–PBS was added to the cells and the reaction mixture was incubated at room temperature for 30 min. Formalin and agar were removed, wells were washed with PBS, and crystal violet solution (0.1% crystal violet in 20% methanol) was added until clear plaques appeared. Wells were rinsed in tap water and the plaques counted. Viremia data are presented as the peak viremia of each mouse at day 1 to day 3 postimmunization or postchallenge.

Foot swelling. Prior to infection and challenge, and at day 4 to day 8 postinfection and postchallenge, the height and breadth of both hind feet were measured using a digital caliper. Foot swelling was calculated as height times breadth of both hind feet, and data are presented as percent foot swelling of each mouse (mean of measurements of both feet) relative to day 0. Peak foot swelling for each mouse is presented.

ELISA. Enzyme-linked immunosorbent assay (ELISA) plates (Maxisorp; Nunc, Odense, Denmark) were coated with 2 μg/ml CHIKV Env p62-E1 protein antigen, in which the ectodomains of CHIKV-115 p62 and E1 are joined with a glycine serine linker to form a soluble protein (34). p62-E1 was expressed and secreted from stably transfected S2 cells as a strep-tagged soluble recombinant protein. The protein was purified on a StrepTrap column (GE Healthcare Life Sciences, Uppsala, Sweden), and the buffer was exchanged to PBS. After 24 h at 4°C, plates were washed with PBS supplemented with 0.05% Tween (Sigma-Aldrich) (PBS-Tween) and blocked with 1% BSA (Gibco/Life Technologies)–PBS for 1 h at room temperature. Sera were diluted in PBS-Tween, added to the plates, and incubated for 24 h at 4°C. Plates were washed, and secondary horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG, IgG1, or IgG2c was diluted 1:5,000 (SouthernBiotech) in PBS-Tween and added, and the reaction mixture was incubated for 1.5 h at room temperature. Plates were washed, and OPD (*o*-phenylenediamine dihydrochloride) substrate (Sigma-Aldrich) was added. HCl (1 M) was added after 15 min to stop the color development. Absorbance was read at 490 nm. The results are presented as endpoint titers.

CHIKV neutralization assay. Titers of neutralizing antibodies were determined as previously described using Chikungunya virus replicon particles (VRPs) expressing Gaussia luciferase (Gluc) (35). Briefly,

BHK-21 cells were seeded in 24-well plates at 10^5 cells per well. The following day, VRPs (MOI of 0.5) were preincubated with 2-fold serial dilutions of serum samples for 1 h at 37°C before the mixture was added to the 24-well plates. After incubation for 1 h at 37°C, the inoculum was removed, cells were washed with PBS, and medium was added. Readout of secreted Gaussia was performed at 24 h postinfection using a renilla luciferase assay system (Promega). Neutralization potency was determined as a percentage of measured Gluc activity compared to the Gluc readout after VRP application without serum. Results are presented as 50% neutralization (NT50) titers.

ELISpot assay. T cell responses were determined by gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assay (Mabtech, Nacka Strand, Sweden) according to the manufacturer's protocol. To obtain single-cell suspensions, fresh spleens were mashed through a 70-μm-pore-size nylon cell strainer (BD). Cells were washed with complete RPMI 1640 (Sigma) supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco/Invitrogen) and then treated with red blood cell lysis buffer (Sigma) for 2 min, washed, and resuspended in complete RPMI medium. Viable splenocytes were counted using a Countess cell counter (Invitrogen), and cells were stimulated with 2.5 μg/ml of a CD8 T cell-restricted E1 epitope (HSMTNAVTI [23]). Results are presented as the number of spot-forming units (SFUs) per 10^6 splenocytes. Responses of at least 25 SFU/10^6 splenocytes and at least four times the background were regarded as representing positive results.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software). A two-tailed Mann-Whitney test was used to analyze differences between two groups, and a Kruskal-Wallis test followed by *post hoc* analysis by Dunn's test was used for multiple comparisons. A Spearman rank test was used to examine correlation between the magnitude of immune responses prechallenge and the level of viremia or foot swelling postchallenge.

Ethics statement. All animal work was conducted in biosafety level 3 (BSL3) laboratories at the Astrid Fagraeus Laboratory, Karolinska Institutet, Sweden, in accordance with the recommendations of the National Board for Laboratory Animals. The protocol was approved by the local ethics committee (Stockholm's Norra Djurförsöksetiska Nämnd; permit number N74/11).

RESULTS

Δ5nsP3 and Δ6K mutants are stable and attenuated *in vitro*. Based on the infectious CHIKV clone LR2006-OPY1 (28), a mutant virus with a large deletion in the C-terminal hypervariable region of nsP3 (Δ5nsP3) and a mutant virus lacking the 6K protein (Δ6K) were constructed. CHIKV and the Δ5nsP3 and Δ6K mutants were successfully produced as viral particles or as infectious DNA plasmids in a previously described DREP vector (32) (Fig. 1A). Titers and plaque morphology of selected viruses measured by plaque assay showed that mutants Δ5nsP3 and Δ6K induced slightly smaller plaques and gave lower titers (1.2×10^7 and 1.7×10^7 PFU/ml, respectively) than CHIKV (4.4×10^8 PFU/ml) (Fig. 1B). Importantly, serial passages of mutants Δ5nsP3 and Δ6K at low MOI on Vero cells did not reveal an increase in titers of collected stocks over five passages, and RT-PCR followed by sequencing showed that the introduced deletions were preserved in all passages of both mutant viruses. We therefore conclude that both the Δ5nsP3 and Δ6K mutants are genetically stable and have reduced replicative capacity *in vitro*.

Infection with CHIKV protects against challenge with a high dose of CHIKV in C57BL/6 mice. A C57BL/6 mouse model (36) was established to study the ability of vaccine candidates to protect against CHIKV infection (Fig. 2A). Here, CHIKV was used for both immunization and challenge. Infection of 10^4, 10^5, or 10^6 PFU CHIKV in the feet showed that 10^6 PFU gave the highest

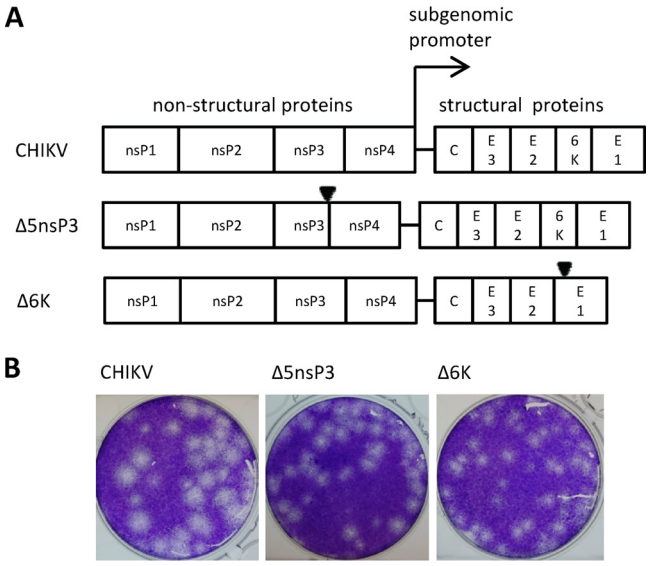

FIG 1 CHIKV vaccine candidates. (A) Schematic figure of the design and characteristics of CHIKV and the vaccine candidates. CHIKV and the Δ5nsP3 and Δ6K mutants were administered either as viral particles or as DNA-launched infectious genomes. Δ5nsP3 has a 183-bp deletion in the 3′ part of the sequence encoding nsP3 (amino acids 1656 to 1717 in the P1234 polyprotein), and Δ6K is devoid of 6K (amino acids 749 to 809 in the structural polyprotein). Locations for deletions are marked with arrows. (B) Plaque morphology of CHIKV and the Δ5nsP3 and Δ6K mutants.

viremia (data not shown), and this dose was therefore used for subsequent challenge studies with the realization that this dose was very high considering the small-animal model. Analysis of viremia and foot swelling showed that they peaked at days 1 to 2 and 5 to 7, respectively (data not shown), confirming published data (36). A single infection with 10^4, 10^5, or 10^6 PFU CHIKV inoculated s.c. in the flank induced similar levels of viremia (Fig. 2B, left panel), but the level of foot swelling did not exceed that seen for naive mice since mice were infected in the flank and not in the feet (Fig. 2C, left panel). Equivalent high IgG titers were induced after infection with the different doses of CHIKV (Fig. 2D, left panel). Postchallenge with 10^6 CHIKV in the feet, none of the previously infected mice showed induction of viremia (Fig. 2B, right panel), and the low levels of foot swelling detected did not differ significantly between the groups of infected mice (Fig. 2C, right panel). Challenge induced IgG titers in noninfected mice similar to those in the previously infected mice (Fig. 2D, right panel). Since CHIKV was able to induce immune responses that protected against challenge with a very high dose, we concluded that this model was suitable to compare the protective efficacy of our vaccine candidates to that induced by CHIKV.

Δ5nsP3 and Δ6K mutants are attenuated *in vivo*. Pathogenicity of vaccine candidates was assessed after a single immunization with 10^5 PFU viral particles or 10 μg DNA in C57BL/6 mice. CHIKV particles induced a level of viremia similar to that seen when establishing the challenge model. No viremia was detected post-intradermal immunization with DNA-launched CHIKV (Fig. 3A); however, viremia was observed post-intramuscular immunization of DNA-launched CHIKV (mean, 10^4 PFU/ml) (data not shown). For the vaccine candidates, mutant Δ5nsP3 induced viremia in two of five mice when given as viral

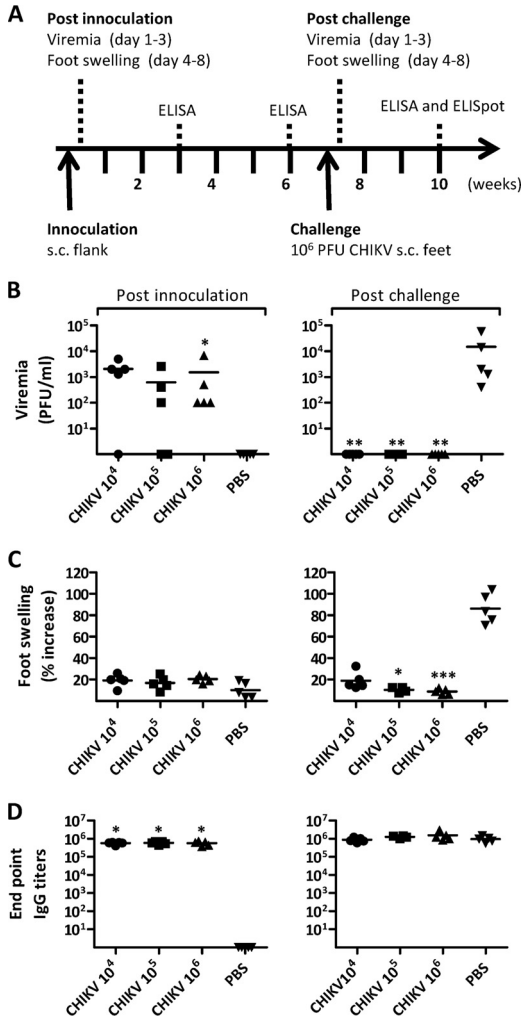

FIG 2 Setup of mouse challenge model. (A) Outline for the challenge study. Female C57BL/6 mice (5 to 6 weeks old) were infected with 10^4, 10^5, or 10^6 PFU CHIKV subcutaneously (s.c.) in the flank and challenged 7 weeks later with 10^6 PFU CHIKV in the feet. (B) Peak viremia titer in serum collected at day 1 to day 3 postimmunization and day 1 to day 3 postchallenge. (C) Peak foot swelling of each mouse (mean of height times breadth of both hind feet relative to day 0) at day 4 to day 8 postimmunization and day 4 to day 8 postchallenge. (D) Endpoint IgG titers at 1 week before and 3 weeks after challenge. Bars show mean values ($n = 5$). A Kruskal-Wallis test followed by Dunn's posttest was used to compare the responses of the different groups. One, two, and three asterisks (*, **, and ***) indicate statistical differences from naive mice (PBS) of $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

particles. However, no viremia was seen in mice infected with 10^4 PFU of the Δ5nsP3 mutant (data not shown), demonstrating that the rather high dose of 10^5 PFU of the Δ5nsP3 mutant accounted for the observed viremia. The high dose of the Δ5nsP3 mutant administered as viral particles in feet did not generate a level of foot swelling significantly higher than that seen with naive mice (Fig. 3B). Mice inoculated with mutant Δ6K or UV-inactivated CHIKV did not show viremia, and the response seen when the vaccine candidates were administered as viral particles in the feet demonstrated no significant foot swelling. The introduced deletions in nsP3 and 6K thus attenuate CHIKV in C57BL/6 mice.

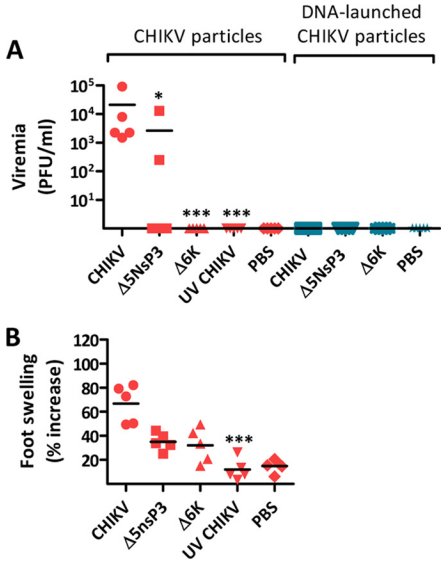

FIG 3 Pathogenicity of vaccine candidates. C57BL/6 mice were immunized with CHIKV or mutants thereof. (A) Peak viremia at day 1 to day 3 after a single immunization with 10^5 PFU viral particles or 10 μg DNA. (B) Peak foot swelling of each mouse (mean of height times breadth of both hind feet relative to day 0) at day 4 to day 8 after a single immunization with 10^5 PFU viral particles in feet. Bars show mean values ($n = 5$). A Kruskal-Wallis test followed by Dunn's posttest was used to compare the responses of the different groups. One asterisk and three asterisks (* and ***) indicate statistical differences from CHIKV of $P < 0.05$ and $P < 0.001$, respectively.

Δ5nsP3 and Δ6K mutants induce strong humoral and cellular immune responses in C57BL/6 mice. Immunogenicity of vaccine candidates was assessed after a single immunization of 10^5 PFU viral particles or 10 μg DNA in C57BL/6 mice. DNA was delivered intradermally followed by *in vivo* electroporation to elicit strong immune responses (32, 37). For antibody responses measured by ELISA, both the Δ5nsP3 and Δ6K mutants induced high IgG titers of a magnitude similar to those seen with CHIKV (10^4 to 10^5) (Fig. 4A), whereas UV-inactivated CHIKV generated no measurable antibody responses. IgG isotyping revealed that antibodies induced by the different vaccine candidates were predominantly isotype IgG2c rather than isotype IgG1 (Fig. 4B), suggesting that the induced immune responses were tilted toward a Th1 type of immune response. Neutralization studies were performed and showed that high (10^2 to 10^4) NT50 titers were elicited by the Δ5nsP3 and Δ6K mutants and that the neutralization titers mirrored the IgG titers for all groups (Fig. 4C). CHIKV and the Δ5nsP3 and Δ6K mutants induced similar high CD8 T cell responses as measured by IFN-γ ELISpot assays (10^3 to 10^4 SFU/10^6 splenocytes), whereas no CD8 T cell responses were detected in mice immunized with UV-inactivated CHIKV (Fig. 4D). Hence, CD8 T cell responses were not affected by the attenuation of the Δ5nsP3 and Δ6K mutants. We conclude that the Δ5nsP3 and Δ6K vaccine candidates elicit strong humoral and cellular immune responses after a single immunization.

Δ5nsP3 and Δ6K mutants protect against challenge with a high dose of CHIKV in C57BL/6 mice. The ability of CHIKV and the various vaccine candidates to confer protection against CHIKV challenge was evaluated. Viremia was measured following challenge with 10^6 PFU CHIKV in the feet and showed that the groups of mice displaying strong immune responses after a single

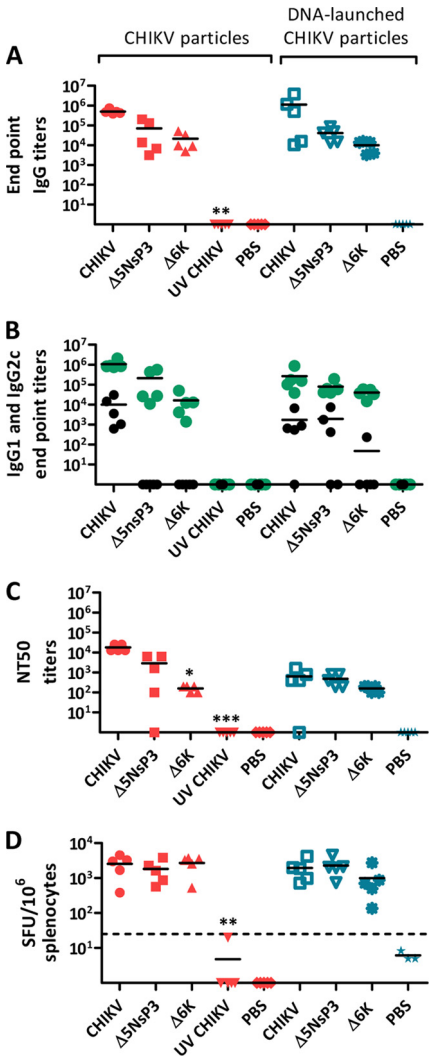

FIG 4 Immunogenicity of vaccine candidates. C57BL/6 mice were immunized once with either 10^5 PFU viral particles or 10 μg DNA. (A to C) Total IgG titers (A), IgG1 (black dots) and IgG2c (green dots) titers (B), and 50% neutralization titers (NT50) (C) on serum collected prior to challenge. (D) CD8 T cell responses measured by an IFN-γ ELISpot assay performed on splenocytes at 7 to 10 days postimmunization. The dotted line represents the cutoff (25 spot-forming units [SFU]/10^6 splenocytes). Bars show mean values ($n = 5$). A Kruskal-Wallis test followed by Dunn's posttest was used to compare the responses of the different groups. One, two, and three asterisks (*, **, and ***) indicate statistical differences from CHIKV of $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

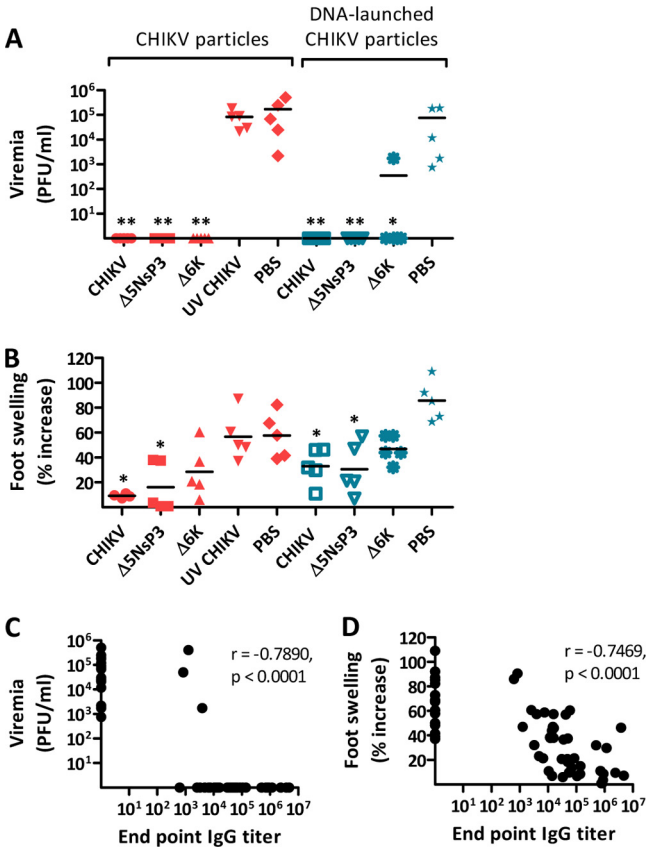

FIG 5 Challenge studies and correlates of protection. (A and B) Peak viremia titer at day 1 to day 3 (A) and peak foot swelling of each mouse (mean of height times breadth of both feet) at day 4 to day 8 (B) postchallenge with 10^6 PFU CHIKV in feet of mice previously immunized with 10^5 PFU viral particles or 10 μg DNA. (C and D) Correlation between endpoint IgG titers before challenge and either peak viremia (C) or peak foot swelling (D) after challenge. Bars show mean values ($n = 5$). A Kruskal-Wallis test followed by Dunn's posttest was used to compare the responses of the different groups, and a Spearman rank test was used to examine the correlation between the magnitude of immune responses prior to challenge and the level of viremia and foot swelling postchallenge. One asterisk and two asterisks (* and **) indicate statistical differences from naive mice (PBS) of $P < 0.05$ and $P < 0.01$, respectively.

immunization were protected and did not develop viremia postchallenge (Fig. 5A). Similarly, even though some foot swelling was detected, potent immune responses prior to challenge apparently restricted subsequent foot swelling on challenge (Fig. 5B). Antibody titers and, to some extent, also CD8 T cell responses were enhanced after challenge (data not shown). Correlates of protection were determined by correlating IgG and NT50 titers postimmunization to viremia and foot swelling in individual mice postchallenge. There was a clear inverse correlation between IgG as well as NT50 titers and both viremia and foot swelling ($P < 0.0001$) (Fig. 5C and D and data not shown). In terms of viremia, the protective IgG titer seems to be 10^3 (Fig. 5C), and the level of CD8 T cell responses detected in splenocytes at day 7 to 10 postimmunization correlated inversely with the viremia and foot swelling seen in mice immunized with the corresponding CHIKV construct in the challenge study ($P < 0.05$) (data not shown). We thus conclude that the strong immune responses induced by a single immunization confer protection against a very high dose of CHIKV infection.

Long-lasting immunity postimmunization with the Δ5nsP3 mutant. To study immunological memory, two sets of mice were immunized once with 10^5 PFU of the Δ5nsP3 mutant and challenged week 8 or 20 postimmunization. IgG titers were equal at week 8 and week 20 postimmunization, and neither viremia nor foot swelling postchallenge differed between the two time points (Fig. 6). Thus, a single immunization with the Δ5nsP3 mutant maintains a long-term memory response and provides long-lasting protection.

Homologous prime-boost immunizations further enhance the potency of the Δ5nsP3 and Δ6K mutants. While realizing the

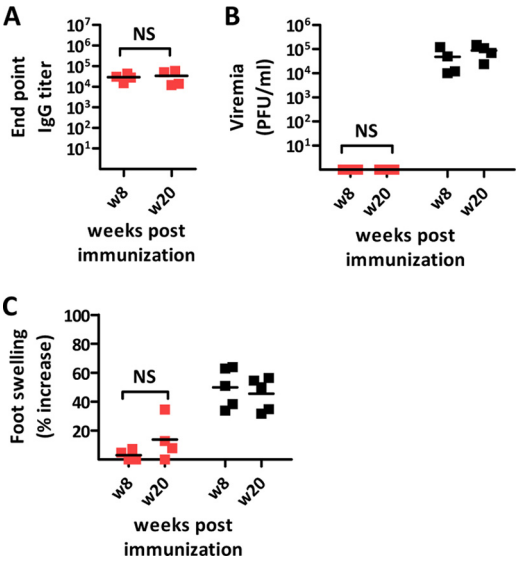

FIG 6 Duration of immunity. Two sets of C57BL/6 mice were injected once with 10^5 PFU Δ5nsP3 (red squares) or PBS (black squares) subcutaneously and challenged with 10^6 PFU CHIKV in the feet at week 8 or week 20. (A) Endpoint IgG titers prior to challenge. (B) Peak viremia at day 1 to day 3 postchallenge. (C) Peak foot swelling of each mouse (mean of height times breadth of both feet) at day 4 to day 8 postchallenge. Bars show mean values ($n = 5$). A two-tailed Mann-Whitney test was used to analyze differences between the two sets of mice. NS, not significant.

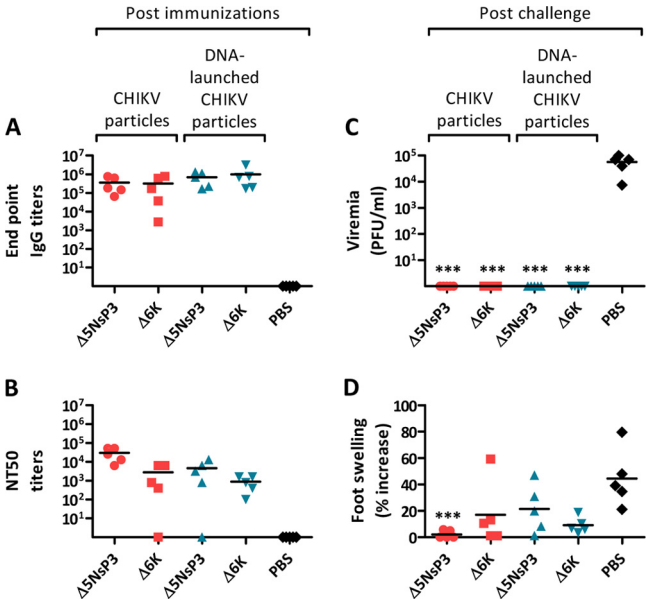

FIG 7 Homologous prime-boost immunizations. C57BL/6 mice were immunized with 10^5 PFU viral particles or 10 μg DNA, with a 3-week immunization interval. Mice were challenged with 10^6 PFU CHIKV in feet at 7 weeks after the second immunization. (A and B) Endpoint IgG titers (A) and 50% neutralization titers (NT50) (B) prior to challenge. (C and D) Peak viremia at day 1 to day 3 (C) and peak foot swelling of each mouse (mean of height times breadth of both feet) at day 4 to day 8 (D) after challenge. Bars show mean values ($n = 5$). A Kruskal-Wallis test followed by Dunn's posttest was used to compare the responses of the different groups. Three asterisks (***) indicate a statistical difference from naive mice (PBS) of $P < 0.001$.

value of a one-shot vaccine, the impact of a second immunization was studied by using 10^5 PFU viral particles or 10 μg of DNA. Homologous prime-boost immunizations with the Δ5nsP3 and Δ6K mutants induced high titers of both IgG and NT50 (Fig. 7A and B) that were approximately 2 orders of magnitude higher than those seen after a single immunization, efficiently protecting against viremia (Fig. 7C). Barely any foot swelling occurred in mice immunized with mutant Δ5nsP3 as viral particles (Fig. 7D), which could be related to the high levels of neutralizing antibodies seen in these mice. We thus conclude that homologous prime-boost can enhance the strong immune response induced after a single immunization and can further reduce foot swelling.

DISCUSSION

We report here the construction and preclinical evaluation of novel attenuated CHIKV vaccine candidates, designed to be delivered either as viral particles or as DNA-launched infectious genomes. The vaccine candidates were genetically stable, highly immunogenic after a single immunization, and able to confer protection against challenge with a very high dose of CHIKV in a mouse model (36).

The rationale for developing an attenuated CHIKV vaccine was to identify a vaccine that could elicit durable immunity after a single immunization that would be similar to the immunity seen after natural CHIKV infection (38). Moreover, an attenuated CHIKV vaccine can easily be produced at biosafety level 2 (BSL-2). The attenuated CHIKV vaccine candidates developed to date rely on a small number of attenuating mutations (11–13) and are at risk of being unstable or even sensitive to reversion. We therefore aimed for a more stable attenuation strategy, choosing to replace a 61-aa region of nsP3 (Δ5nsP3) with a short linker or to delete the entire 6K (Δ6K). Indeed, no genetic alterations were detected after serial passage of these engineered viruses, suggesting that the risk of reversion to virulence is very unlikely. Furthermore, when we deleted the gene encoding 6K, we also prevented the production of the recently identified TF protein that can be expressed during a frameshift event during translation of 6K (39). Similar to 6K, the TF protein is important for the formation and release of CHIKV and other alphaviruses. Hence, the Δ6K mutant lacks yet another CHIKV protein associated with pathogenesis, which further enhances the safety profile of this vaccine candidate.

Although attenuated vaccines in general represent powerful vaccine tools, they can generate adverse effects (25). For the Δ5nsP3 and Δ6K mutants evaluated in this study, the replicative capacity of both was clearly reduced *in vitro* as evidenced by a reduction of more than 1 log in titers and a smaller plaque size than CHIKV, similar to observations reported for SFV mutants with the corresponding deletions (29, 30). *In vivo*, 10^5 PFU of the Δ5nsP3 mutant induced low but detectable viremia in two of five C57BL/6 mice. However, no viremia was detected after immunization with 10^4 PFU of the Δ5nsP3 mutant, suggesting that the observed reactogenicity can be explained by the rather high vaccine dose of 10^5 PFU used in this study. Accordingly, considering that humans are very much larger than mice and that the 10^5 PFU dose would be used for humans as well, we hypothesize that no adverse events would be seen in clinical trials.

Despite the attenuated profile of the Δ5nsP3 and Δ6K mutants, both induced strong T cell and antibody responses after a single immunization that were not significantly different from those seen with CHIKV. Moreover, the NT50 titers of 10^2 to 10^4 elicited

by the mutants are to be considered potent compared to neutralizing antibody titers induced by other CHIKV vaccine candidates in mouse models (11–13, 15–19, 21–24). Similar to previous observations of CHIKV infection in C57BL/6 mice, mainly IgG2c antibodies were induced (36, 40), demonstrating that CHIKV infection induces a Th1-biased immune response such as is usually required to combat viral infections.

Overall, the Δ5nsP3 and Δ6K mutants encoded by DNA induced immune responses comparable to those induced by the mutants administered as viral particles, enabling us to us to evaluate the most feasible vaccine modality for a certain setting. The lack of antibody and T cell responses after immunization with UV-inactivated CHIKV was probably due to the rather low dose that was used to match the titer of the Δ5nsP3 and Δ6K mutants. Considering that the volume of 10^5 particles of inactivated CHIKV used in this study roughly corresponds to a total weight of 0.01 ng, this is a dose a million times lower than the 10 μg of inactivated CHIKV required to induce detectable antibody responses after a single immunization in mice (36) and is thus too low to elicit an immune response. Accordingly, the strong immune response elicited by the relatively low titers (i.e., small amount of antigen) of the Δ5nsP3 and Δ6K mutants highlights the benefit of attenuated over inactivated and subunit vaccines that typically require high antigen doses to elicit potent immune responses.

Several mouse models have been used to study CHIKV infection (reviewed in reference 41), including mice devoid of type 1 interferon or CD8 T cell activity (42, 43). We and others have used s.c. infection with CHIKV in the feet of C57BL/6 mice, since this model has a fully functional immune system and resembles the route of infection and pathogenicity seen in humans (36). By studying the vaccine candidates in this extreme challenge model, we demonstrated that a single immunization with the Δ5nsP3 and Δ6K mutants protected against viremia and, to a good extent, foot swelling. We also showed that there was a clear inverse correlation between binding and neutralizing antibody titers prior to challenge and both viremia and foot swelling postchallenge. This supports recent studies highlighting the importance of antibodies in protection against CHIKV in both mice (40, 44) and humans (38). Since antibodies correlated with protection against challenge, and as mainly IgG2c antibodies were induced, we conclude that IgG2c is the IgG isotype that confers protection. Although T cells have been suggested to play only a limited role in protection against CHIKV infection (44), both CD4 and CD8 T cell subsets are required to develop functional and long-lived immune responses and to clear CHIKV-infected cells, respectively (40, 44, 45). Hence, the strong T cell response elicited by the Δ5nsP3 mutant probably plays a significant role in the formation of the long-lived protective antibody response that was observed in the memory study.

Although the aim was to develop a CHIKV vaccine that induced protective immunity after a single immunization, homologous prime-boost immunizations were studied and showed that the strong binding and neutralizing antibody responses generated by a single immunization were further enhanced by approximately 2 orders of magnitude after a second immunization and that the mice were fully protected from viremia upon challenge. Moreover, the second immunization further reduced foot swelling on challenge, especially in mice immunized with mutant Δ5nsP3 that had NT50 titers of 10^4 to 10^5, where barely any foot swelling was observed. This low level of foot swelling was otherwise observed only after a single immunization with CHIKV, holding promise for these vaccine candidates in prime-boost immunization strategies. Regardless of immunization schedule and induced antibody titer, a low level of foot swelling was always seen after challenge, even postinfection with CHIKV. Similar to the low level of foot swelling seen postinjection with 10^5 PFU of the Δ5nsP3 and Δ6K mutants in feet, this might have been due to the very high dose of 10^6 PFU CHIKV used for challenge, corresponding to 10^5 50% monkey infectious doses (MID50) (46), which was considerably higher than the challenge doses used in other CHIKV vaccine studies where mice were challenged in feet (10^2 to 10^4 PFU) (12, 13, 18, 44).

In summary, this report demonstrates the construction of stable, highly immunogenic, and efficient attenuated CHIKV vaccine candidates that can be administered either as viral particles or as infectious genomes launched by DNA. The potency of these vaccine candidates will be further studied in nonhuman primates.

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